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Rapid Detection of Anticardiolipin Antibodies

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A rapid screening method for the detection of antiphospholipid antibodies is described. Dense, red dyed polystyrene beads coated with cardiolipin were incubated with test sera for a short period of time, then added to a microtube containing anti-human IgG in a gel provided within a pre-cast card (DiaMed ID Microtyping System). The card was centrifuged at 150*g* for 5 min and then examined for movement of the beads through the gel. Beads without bound antibody travelled through the gel and formed a pellet on the bottom of the tube. Anti-human IgG within the gel matrix impeded cardiolipin-coated beads when antiphospholipid antibodies bound to the beads. Positivity was indicated by the formation of a layer of beads on the top of the gel matrix. Prospective analysis of 103 samples for the presence of antiphospholipid antibodies by flow cytometry and the gel-card technique showed good correlation between the two methods. All samples found to be positive by flow cytometry (23 of 103) were identified as positive by the gel-card technique. Two samples were identified as positive by the gel-card method but negative by flow cytometry. The technique is simple to perform and should prove useful as a rapid screening method for the detection of antiphospholipid antibodies. Am. J. Hematol. 57: 315–319, 1998.

Key words: antiphospholipid antibody; anticardiolipin antibody; phospholipid; microsphere

INTRODUCTION

Antiphospholipid antibodies (APA) are autoantibodies that bind phospholipid directly [1–3] or phospholipid in combination with specific cofactor proteins [4–7]. The antibodies have been associated with thrombocytopenia, thrombosis (venous and arterial), and recurrent fetal loss [8].

Detection of APA in serum or plasma samples can be accomplished using enzyme-linked immunosorbent assay (ELISA) procedures [9] or a recently described flow cytometric method based on antibody binding to phospholipid-coated polystyrene microspheres [10]. These procedures are suited for testing large numbers of samples and providing quantitative and/or semi-quantitative results. Recent studies have shown that approximately 30% of samples referred for a thrombotic assessment are found to be positive for APA [11]. A simple screening procedure that accurately identifies the presence of APA in samples, allowing selective quantitation by ELISA or flow cytometric methods, would permit considerable time and cost savings.

Lapierre et al. [12] described a simple method for the identification of anti-red cell antibodies in test sera prior to red cell transfusion. Recipient sera are incubated with test red cells, then added to the top of a DiaMed ID Microtyping card. The gel is spun in a swing-out centrifuge to force the red cells through the gel matrix. Anti-red cell antibodies in the test sera having bound to the red cells are captured by the anti-human IgG in the gel and impede the movement of cells through the gel. Positive samples are indicated by a layer of red cells on top of or within the gel while negative samples display a button of red cells at the bottom of the microtube.

We hypothesized that by utilizing the phospholipid-coated polystyrene beads technology described by Stewart et al. [10] and the impedance of antibody-coated cells by gel matrix described by Lapierre et al. [12], a simple, rapid screen for the presence of APA could be developed.

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This was accomplished using high-density particles described by Hobbs et al. [13] and Schwind et al. [14].

MATERIALS AND METHODS

Preparation of Phospholipid-Coated Beads: Gel Tube Method

Dyed (blue, violet, red, or yellow) polystyrene beads (1.0, 3.0, or 6.0 μm; 1.05 g/cm^3) were purchased from Polysciences Corporation (Warrington, PA). Red-dyed polystyrene beads (originally described by Hobbs et al. [13]) of higher density (diameter = 3 μm; 1.30 g/cm^3) were supplied by DiaMed AG (Switzerland). The beads were coated with cardiolipin (CL) (Sigma, St. Louis, MO) as previously described [10]. Briefly, 1×10^8 beads were washed with absolute ethanol (BDH, Edmonton, AB) three times by centrifugation and finally resuspended in cardiolipin/ethanol (2 mg/ml; final concentration). The suspension was incubated overnight at 4°C in the dark. Beads (1×10^7) were removed to a separate tube and diluted with tris buffered saline (TBS; 0.01 M, pH 7.2) and centrifuged to a pellet. The supernatant was aspirated to waste and the beads resuspended in 1 ml 10% (v/v) fetal calf serum (FCS; Gibco/BRL, Burlington, Ontario) and incubated at 37°C for 15 min. The beads were then left at room temperature until needed.

Samples

Serum samples referred to the hematology laboratory for investigation of patients with possible antiphospholipid syndrome were tested by both the phospholipid-bead flow cytometry method and the anti-globulin gel test protocol as described below.

Flow Cytometry

Testing was conducted as previously described [10]. Briefly, 4-μm polystyrene beads (Polysciences) were coated with CL and blocked with 10% FCS as outlined above. Test sera were added to the blocked beads to yield a final serum dilution of 1/100. The samples were incubated for 15 min, then spun to a pellet and resuspended in a cocktail of saturating goat anti-human IgG f(ab′)2 conjugated to phycoerythrin (PE) (Zymed Laboratories, San Francisco, CA) and goat anti-human IgM f(ab′)2 conjugated to fluorescein isothiocyanate (FITC) (Zymed) and incubated a further 15 min. Samples were analysed on a FACScan flow cytometer (Becton Dickinson, Mountainview, CA). The samples were graded as negative, low-, medium-, or high-positive based on comparison to the fluorescence intensity of standard samples of known APA concentration (Louisville APL Diagnostics, Louisville, KY) binding to the CL-beads. Negative samples were considered to have less than 10 U/ml GPL, low-positive samples 10–20 U/ml GPL, medium-positive samples 21–80 U/ml GPL, and high positive samples >80 U/ml GPL. A unit of GPL (i.e., IgG binding to phospholipid) activity is defined as 1 μg of IgG binding cardiolipin in 1 ml [15].

TABLE I. Gel Test Protocols

Bead	Centrifugation (min)	Pos/neg Discrimination
1 μm (1.05 g/cm^3)	5, 150*g*	Poor
	10, 150*g*	Good discrimination of high positive from negative
	10, 400*g*	Poor (false positives)
3 μm (1.05 g/cm^3)	5, 150*g*	Good discrimination of high positive from negative
	10, 150*g*	Poor (false positives)
	10, 400*g*	Poor (false positives)
3 μm (1.30 g/cm^3)	5, 150*g*	Good discrimination of moderately positive from negative
	10, 150*g*	Good discrimination of high positive from negative
6 μm (1.05 g/cm^3)	5, 150*g*	Poor

Gel Test Protocol

Serum samples with known APA levels were used to optimize the conditions of the anti-globulin gel method. Broad discrimination between negative and high positive samples were evaluated to define the most appropriate type of bead to use and the incubation and centrifugation conditions. The anti-globulin gel cards (anti-IgG; DiaMed AG, Cressier Sur Morat, Switzerland) were centrifuged in a swing-out rotor at various forces and time periods using a Beckman (Palo Alto, CA) GPR tabletop centrifuge. Samples were considered negative if a pellet of beads was observed at the bottom of the gel-tube, post centrifugation. Positive samples were defined by a layer of beads on the top of or within the gel after centrifugation and/or the lack of pellet formation at the bottom of the gel tube.

RESULTS

Optimizing the Gel Test Protocol

Incubation times and centrifugation steps were varied to optimize discrimination between positive and negative samples using the various beads listed in Table I. CL-coated beads of 6-μm diameter showed poor penetration into the gel, even in the absence of a serum sample. CL microspheres of 1-μm diameter (1.05 g/cm^3) provided clear differentiation between negative and high positive samples with a 10-min centrifugation step at 400*g;* however, low and moderately positive samples gave varying responses. The denser red-dyed beads (1.30 g/cm^3) coated with CL provided the best discrimination between negative and low or moderately positive samples (Figs. 1

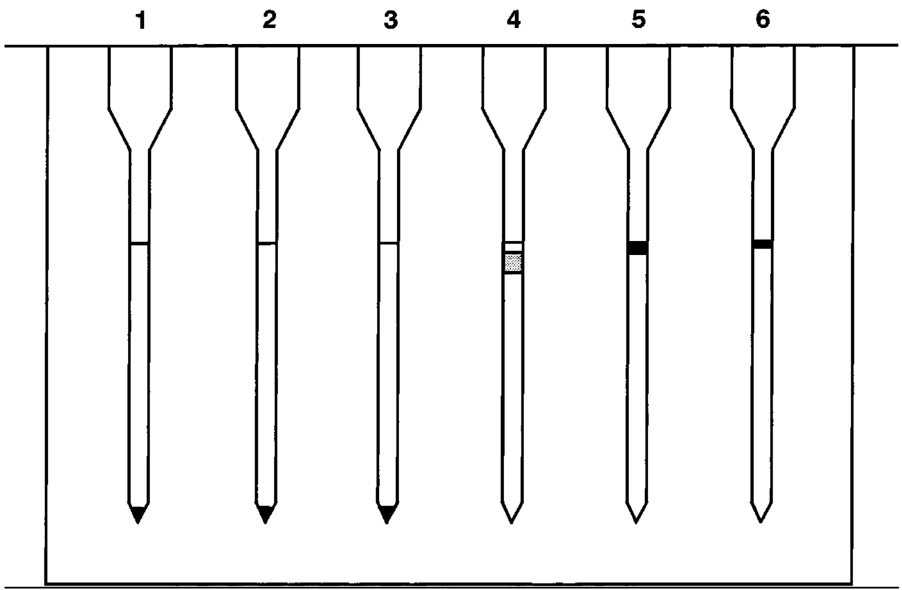

Fig. 1. Diagrammatic representation of ACA screening results using dense (1.30 g/cm^3) red beads. 1, Native red beads; 2, CL-coated red beads blocked with 10% FCS; 3, CL-coated red beads + negative sample; 4, CL-coated red beads + low positive sample; 5, CL-coated red beads + medium positive sample; 6, CL-coated red beads + high positive sample. Negative <10 U/ml GPL, low positive 10–20 U/ml GPL, medium positive 21–80 U/ml GPL, high positive >80 U/ml GPL.

and 2). The following conditions were, therefore, selected for prospective analysis of patient samples: The CL-coated beads (1×10^7 ml, 10 μl) were loaded into each microtube. Serum samples (1 μl) were added to the bead layer and mixed by aspiration. The cards were incubated in an upright position for 5 min at RT, then placed in specially designed adapters in a Beckman GPR centrifuge equipped with a swing-out rotor. Centrifugation was carried out for 5 min at 150*g* at RT (no brake).

Flow Cytometry Vs. Gel Test Detection of APA

One hundred and three serum samples were prospectively analysed by phospholipid-bead flow cytometry and the gel test method. Samples were graded as negative, low-, medium-, or high-positive (flow cytometry); or negative or positive (gel-tube). Although the clinical relevance of a low positive result (i.e., <10 U/ml, established for our laboratory) has yet to be established, this

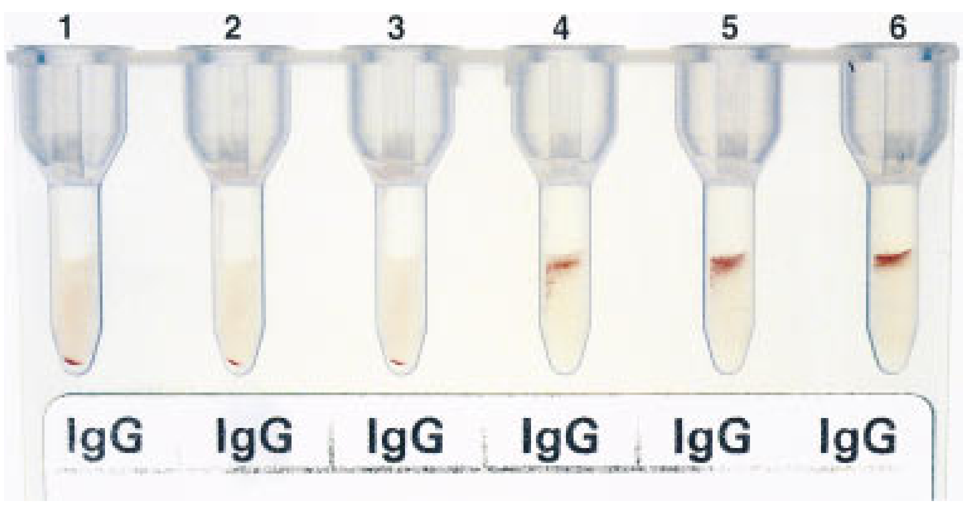

Fig. 2. ACA screening results (photograph). Ten-microliter CL-coated dense red beads (1×10^7/ml) were incubated with 1 μl test serum (where applicable) for 5 min in the bowl of the anti-IgG microtube. The tops of the cards were sealed with cellophane tape and spun at 150*g* for 5 min at RT using a swing-out rotor containing specific adapters for the gel test cards. The microtube numbers correspond to the conditions outlined in Figure 1. Note the tight band of beads in tube 6 (high positive) vs. the loose layer of beads penetrating the gel in tube 4 (low positive). Magnification ×2.2.

TABLE II. Comparison of Flow Cytometry With Gel-Tube Detection of ACA

	Flow cytometry	Gel test
GPL+ (n = 23)	23	23
MPL+ (n = 11)	11	0
GPL− (n = 67)	67	65 (2 positive)

level of ACA was chosen to discriminate between positive and negative samples to ensure that all samples with ACA-associated thrombocytopenia and/or thrombosis would be detected. Sensitivity of the gel test method was confirmed, retrospectively, by repeat analysis of low positive samples (N = 10), all demonstrating positivity as indicated by a layer of beads on the top of the gel matrix, post centrifugation. As shown in Table II, 25 of 103 samples analysed prospectively, were found positive by the gel test method, while 23 samples were found positive for IgG binding by the flow cytometry procedure. All samples found positive by the flow cytometry procedure were also found positive by the gel test method. Two samples negative by the flow cytometry procedure were found to be positive by the gel test method. The samples ranged in GPL positivity as follows: 3 low positive (10 to 20 U/ml), 10 medium positive (21 to 80 U/ml), 10 high positive (>80 U/ml). It should be noted that no samples found positive for MPL ACA (flow cytometry) but negative for GPL, showed positivity in the gel test method (samples positive only for MPL by flow cytometry ranged from 10 to 60 U/ml, N = 11).

DISCUSSION

Investigation of patients who present with thrombotic tendencies may require evaluation of low or dysfunctional levels of natural anticoagulants (protein C, protein S, antithrombin III) or screening for Factor V_{Leiden}. Screening for the presence of antiphospholipid antibodies is also an essential part of a pro-thrombotic work-up. However, even a select population of patients such as those who tend to thrombose have a moderately low incidence of positivity (approximately 30%) [11]. Rapid, inexpensive screening of samples for the presence of antiphospholipid antibodies would translate into considerable cost savings in both reagent and labor costs.

Detection of antiphospholipid antibodies using phospholipid-coated polystyrene microspheres by flow cytometry enables simultaneous evaluation of several phospholipids (as solid phase antigens) and several antibody isotypes at one time [10]. The phospholipid-coated beads are very stable when kept in ethanol (>3 years with coefficients of variation less than 10%, data not presented). Superior sensitivity and specificity of the phospholipid bead flow cytometry procedure in comparison to the anticardiolipin ELISA bench method has recently been established in a wet workshop conducted during the VIIth International Symposium on Antiphospholipid Antibodies [16]. However, access to flow cytometry facilities may be limited to specialized laboratories.

Screening for the presence of recipient antibodies to donor red cells was originally described by Lapierre et al. [12] and is now offered commercially in the form of a plastic card consisting of six microtubes containing antihuman globulin suspended in the gel (DiaMed AG). The results of the test are scored by eye or by means of an ID density reader; positive results are indicated by a layer of donor red cells on the top of or within the gel and negative results are indicated by a pellet at the bottom of the gel. The red cell indicator system is very efficient given the density of the red cell (1.0928–1.1000 g/cm^3) [17] and the natural ability of the RBC to deform (i.e., movement through capillary beds). The same centrifugation conditions for the red cell antibody screen (85*g*, 10 min) were found unsuitable for the bead-based screen for anticardiolipin antibodies. Given the non-deformable nature of the beads, a compromise between bead density, and time and force of centrifugation had to be achieved. For example, centrifugation of the gel-cards at high speed resulted in gel packing, which inhibited movement of the beads through the gel.

The red-dyed beads of higher density (1.30 g/cm^3) proved to be the optimal solid phase support for coating with CL, and discrimination between APA-negative and -positive samples. Smaller beads presumed to achieve better penetration into the gel, required higher centrifugal force to spin these beads of less density (1.05 g/cm^3) through the gel and resulted in gel packing and poor discrimination between negative and positive samples.

Samples positive only for MPL ACA did not display false positivity, confirming antibody isotype specificity of the anti-IgG gel test method. However, two samples found negative for both GPL and MPL by flow cytometry were found to be positive by the gel-tube method. Repeat testing on three separate occasions confirmed these results. Both patients had unremarkable clinical histories (bacterial sepsis and uremia, respectively). Although we are at a loss to explain these results, it should be emphasized that all samples found positive by flow cytometry were also found positive by the gel-tube method (i.e., no false-negative results). Unfortunately, the majority of samples tested were referred from other laboratories within the region, making it difficult to correlate the clinical status of the patient with the test results.

CONCLUSIONS

The gel test system in conjunction with the phospholipid bead technology appears to be an ideal means of rapidly screening for the presence of anticardiolipin an-

tibodies. Testing is simple to perform and evaluate. The equipment required is relatively inexpensive. The test in its present form is suitable for detecting ACA IgG with subsequent quantitation by established methods [9,10]. Given the increasing interest in the evaluation of patients with hyper-coagulable states, this method provides a rapid, cost-effective means of screening samples.

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